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Protein Extraction from *Spirulina platensis* by Using Ultrasound Assisted Extraction: Effect of Solvent Types and Extraction Time

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Abstract. Protein is a substantial nutrition that essentially required by human. *Spirulina platensis* (*Spp*), well known as protein source could be a significant source to be used for many industrial applications. This study was investigated the effectiveness of ultrasound assisted extraction (UAE) method for protein extraction from *Spp* at various composition of solvent mixture and extraction time. Ethanol and mixture of methanol-ethanol were used as solvent. Extraction was conducted by varying ratios of solvent to biomass at 10:1, 12.5:1, and 15:1 (v/w), and extraction time (20, 35, and 50 min). Optimum protein recovery from dry *Spp* was 42.55 ± 0.43% obtained by using 20 ml of the mixture of methanol and ethanol at 50 min of extraction time. This study also conducted that mixture of methanol and ethanol was a better solvent on improving the ultrasound assisted extraction, as indicated by high protein recovery with less amount of solvent volume used.

Introduction

During the twenty-first century, the world population is projected to increase rapidly to 2.3 billion people in 2050; that requires 70% increase of current food production fulfill this fast growth [1]. Massive efforts to reduce global hunger has been initiated by the improvement of food production in the agriculture sector, relatively with the mean per capita income [2]. However, 50% of about 10-11 million children under 5 years in developing countries die every year because of malnutrition [3]. Protein could play important roles for nutritional food that is essential in developing human body system. Source of protein can be found in several diary and poultry product, also other processing form food farm and agriculture industry. Among the protein sources, *Spirulina platensis* (*Spp)* demonstrates the potential as candidate of main protein source. It is indicated by its protein content has high range from 46-63% [4]. In addition, it is also enriched by various essential acids including histidine, threonine, methionine, valine, lysine, cysteine, and other non-essential amino acids [5]. This has drawn huge attention for researchers to study the application of the protein from *Spp* into many such as biofuel feedstock, human food supplement, medication, and also anti-oxidation activity[4, 6-8]. Furthermore, *Spp* has been applied as a dietary supplement to provide insufficient nourishment of poor children in developing countries [9].

The greatest challenge in *Spp* application is how to isolate the protein effectively in the most efficient way. Previous studies reported the isolation of protein was carried out by different cell disruption methods such as chemical, physical, and enzymatic treatments [2]. Chemical treatment use detergents, solvents, and antibiotics, while physical treatments are for example bead milling/grinding, high pressure homogenization, and ultrasonication [10], and there are also enzymatic treatments (e.g. polysaccharidase degradation) [2]. The high-pressure homogenization and chemical treatment have been isolated more protein yield than ultrasonication and manual grinding [11]. However, some high-pressure methods like the French press or Hughes press can be used only in the laboratory scale. Chemical methods are also found to be associated with toxic for human health and the environment [12]. Enzymatic treatment required high cost and low efficiency [13].

Recently, protein extraction using ultrasound assisted extraction (UAE) has been widely conducted on microalgae species, particularly *Spp* [4-8, 10, 11]. This method provides shorter extraction time, energy-saving, low temperature, high yield recovery, and low extraction cost [12, 13]. Therefore, it will a promising option to extract protein from *Spp* in the best manner. Yields of extraction in UAE are affected by particle sizes, types of solvent, temperature, solvent to biomass ratios, and time of extraction are critical parameters in the UAE method to obtain high yields [14]. Different types have been employed to extraction the protein, include methanol, mixture of methanol and ethanol [5], distilled water [6, 10], and sodium phosphate buffer [7]. In this paper, we aimed to study the effect on different solvent types, ratios of solvent to biomass, and extraction time with the UAE of *Spp* to determine the relatively best condition for protein recovery.

Materials and Method

Microalgae. Dry biomass of *Spp* was obtained from Nogotirto Algae Park (Yogyakarta, Indonesia) and stored in refrigeration under the temperature of 4.15 ± 0.07 ℃ for the experiment. The dry *Spp* has 10.73 ± 0.04% (w/w) of moisture content and 49.515 ± 0.33% (w/w) of protein content.

Reagents. Ethanol (96%, reagent grade) and methanol (96%, reagent grade) were purchased from Merck. Lowry protein kits including protein standard, Folin-Ciocalteu phenol, and Lowry reagent were obtained form Sigma Aldrich and used to analyze protein isolated.

Ultrasonic apparatus. In this experiment, ultrasonic bath (100 W Ultrasonic transducers, China) was used for ultrasound assisted extraction (UAE). Extraction processes were conducted at frequency of 25 kHz. During the operation, ultrasound waves release the cavitation bubble with shear force to disrupt the *Spp* cells.

Protein extraction process. Proteins were extracted using with two different solvents: methanol-ethanol mixture (ratio 2:1 v/v) and ethanol. In this experiment, 2 g of *Spp* were transferred into 125 ml conical flash and added with 20 ml of solvents, for each type in two separate flasks. Other experiments were also prepared simultaneously by adding 25 and 30 ml of solvents. Mixtures were then covered by aluminum foil and stirred with magnetic stirrer for 30 min at temperature 25 ℃. The samples were transferred to ultrasonic bath and underwent UAE for 20, 35, and 50 min. The mixtures were separated by using filter paper. The supernatants were then kept for protein analysis.

Protein analysis. The supernatant of filtered sample were used to analyze the protein concentration by Lowry method [15]. For this method, 1 ml of each protein standard concentration (50, 100, 200, 300, and 400 µg/ml) was used for preparation of a calibration curve. Blank was prepared 1 ml of distilled water in a test tube and 0.1 ml of each sample was prepared by diluting with 1 ml distilled water.

Each tube of samples, standard solution, and blank were added with 1 ml Lowry reagent and allowed to stand at room temperature for 20 min. Each sample were immediately mixed with 0.5 ml of Folin-Ciocaltue phenol reagent and incubated for 30 min at room temperature for color development. After incubation, each sample were added with 1 ml of distilled water because the cuvette requires more than 2.5 ml to be analyzed optical density (OD). The absorbance of diluted blue color solution was then determined at 750 nm with a UV-VIS RS spectrophotometer. The protein concentrations were determined from calibration curve by multiplying with their dilution factors to obtain the concentration in original samples and protein content in *Spp* was calculated by (Eq. 1):

$$Protein\ (\%) = \frac{Protein\ concentration \times V}{m_o} \quad (1)$$

where V is the extract volume (ml) and m_o is the dry mass of *Spp*. The protein recovery was determined by (Eq. 2):

$$Protein\ recovery\ (\%) = \frac{Protein\ (\%)}{Total\ protein\ in\ dry\ mass} \times 100 \quad (2)$$

where the total protein in the dry biomass of *Spp* is 49.515 ± 0.33% (w/w).

Data analysis. In this experiment, the protein recovery from the extraction of *Spp* were compared according to different solvent and extraction time, which were expressed by the mean ± standard deviation. ANOVA test was also conducted by analyzing 2 replications to determine the significant different at $P < 0.05$.

Results and Discussion

Effects of solvents on the extraction of protein. Fig. 1(A) shows the protein recovery from UAE using mixture of methanol and ethanol (Me: Eth) at various extraction time. The highest protein recovery (42.55 ± 0.43%) was achieved at 20 ml of the mixture (Me:Eth) within 50 min of extraction time, while the lowest (21.54 ± 1.61%) was at 25 ml of the mixture (Me:Eth) within 20 min of extraction time. Fig. 1(B) shows the protein recovery from UAE using different volumes of ethanol and extraction time. By using 30 ml of ethanol and 50 min of extraction time, the highest protein recovery was obtained at 30.20 ± 2.79% when using 25 ml of ethanol at 35 min, the lowest was at 8.38 ± 0.89% ($P < 0.05$). Solvent is one of the critical parameters on UAE to extract bioactive compounds from microalgae [14], such as protein from *Spp*. A higher amount of solvent could not improve the solubility of analytes but it led to decreasing protein recovery due to excessive solvent. Moreover, the excessive solvent potentially changed solute solubility and altered chemical equilibrium [5]. As shown in Fig. 1(A), using 25 and 30 ml of the Me: Eth mixture could not improve the recovered protein yield. Furthermore, the amount of protein extracted by the mixture of Me: Eth in this experiment was significantly higher than using a single solvent as ethanol ($P < 0.05$). The choice of solvent types is based on polarity, surface tension, viscosity, and vapour pressure. In this context, ethanol and methanol are polar solvents but the mixture of both solvents could extract more protein yield than a single polar solvent because ethanol play an important role to extract protein and methanol could assist better extraction performance [16]. Hadiyanto et al [17] have used ethanol to extract phycocyanin. Phycocyanin has been known as a pigment compound from phycobiliprotein as protein compound of microalgae. Moreover, using the mixture of methanol and ethanol for protein extraction have been also described to lead greater yield than methanol as the single polar solvent [5].

Effect of extraction time on the extraction of protein. UAE utilizes acoustic power to release bubbles cavitation with high shear forces for disrupting the cell wall of plant material to allow the permeability of solvent and for improving the contact surface area of the solvent and compound of interest [18]. Extraction time is a main effective parameter in the UAE process when using short extraction time could not give a complete extraction [14]. It could be seen in Fig. 1(A) and 1(B) with lower protein recovery by using 20 and 35 min of extraction time, while using 50 min of extraction with the mixture (Me: Eth) and ethanol used in this process was recovered higher protein yield. Extraction using a short time is affected by entire extraction and extraction using a long time could have the release of undesirable reactions.

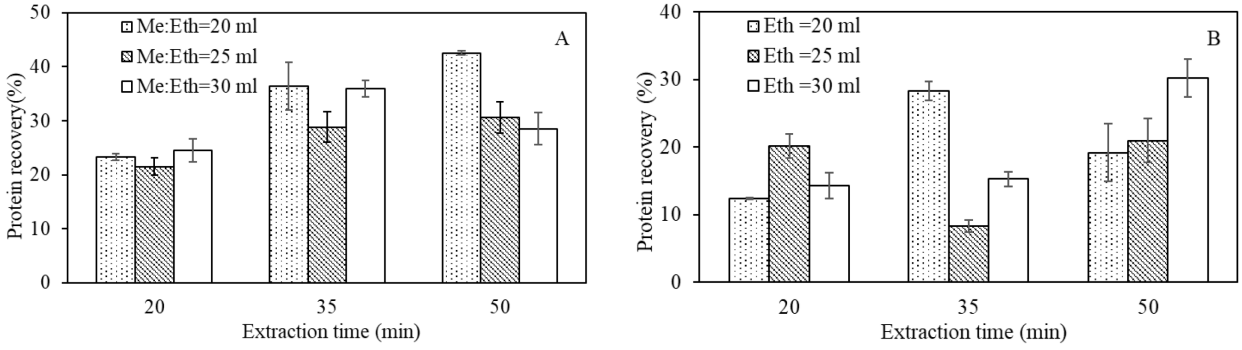

Fig. 1 Protein recovery obtained from different extraction time:
(A) mixture of methanol and ethanol, and (B) ethanol.

Table 1 shows the comparison of previous researches on the protein extraction from *Spp.* Hadiyanto and Adetya (2018) was used only 30 min with the mixture of Me:Eth to obtain the optimum protein yield at 43.96% [5], which is significantly higher than the current research only at 21.06%. However, it could be identified that the high variation of these two studies causes from the different sample used and the frequency of ultrasonic. This previous study used wet biomass of *Spp* with high ultrasonic frequency at 40 kHz, while the recent study used the dry basis of *Spp* with lower frequency only at 25 kHz. Hence, the amount of protein content in *Spp* could be also different to distinguish the results of protein yield. Safi et al (2014) used ultrasonication with distilled water to obtain higher protein yield (47.10 %) but this previous study requires high energy due to the freeze drying process [10], while the recent study used conventional sun drying process. However, the protein from dry *Spp* in the recent study could be different from the previous study. In addition, the recent study used ultrasonic bath to avoid the evaporation of solvent during extraction process.

Table 1. Comparison of optimum protein yield from microalga for selected extraction processes

Substrate	Method	Solvent	Optimum protein yield (%)	Optimum condition	Reference
Spirulina platensis (*Spp*)	Ultrasound assisted extraction (UAE)	Methanol-ethanol (2:1)	21.06 ± 0.21 (dw)	10:1 ratio of solvent to biomass (v/w), 50 min extraction time	This study
Spp	Ultrasound assisted osmotic shock method	Methanol-ethanol (2:1)	43.96 (wb)	15.12% NaCl, 10:1 ratio of solvent to biomass (v/w), 30 min extraction time	[5]
Spp	UAE	Distilled water	29.05 (dw)	Temperature 45 °C, pH 7.46, and time 120	[6]
Spp	Manothermosonication (MTS)	Sodium phosphate buffer	28.42 (dw)	20 min of MTS treatment	[7]
Spp	High pressure cell disruption Chemical treatment Ultrasonication Manual Grinding	Distilled water	78.0 (dw) 53.40 (dw) 47.10 (dw) 35.0 (dw)	50:1 ratio of solvent to biomass (v/w) with 2700 bar 50:1 ratio of solvent to biomass (v/w) with NaOH and HCl 50:1 ratio of solvent to biomass (v/w) with 30 min extraction time 50:1 ratio of solvent to biomass (v/w), 2 h extraction time	[10]
Spp	Ultrasound and Mechanical agitation	Purified water and NaOH	75.76 pr (dw)	33 min sonication and 40 min agitation time	[4]

dw: Dry weight; wb: Wet basis; pr: protein recovery

Conclusion

In conclusion, the protein extraction from UAE by using the mixture of methanol and ethanol could improve the yield of protein recovery and reduce the amount of solvent used. In addition, further studies are needed to investigate various ultrasonic frequencies in order to reduce extraction time and possibly improve protein yield.

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